

An Improved Method for Clearing and Staining Free-hand Sections and Whole-mount Samples*

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Received: 17 March 2005 Returned for revision: 25 May 2005 Accepted: 10 August 2005 Published electronically: 28 September 2005

• **Background and Aims** Free-hand sectioning of living plant tissues allows fast microscopic observation of internal structures. The aim of this study was to improve the quality of preparations from roots with suberized cell walls. A whole-mount procedure that enables visualization of exo- and endodermal cells along the root axis was also established.

• **Methods** Free-hand sections were cleared with lactic acid saturated with chloral hydrate, and observed with or without post-staining in toluidine blue O or aniline blue. Both white light and UV light were used for observation. Lactic acid was also used as a solvent for berberine, and fluoro yellow for clearing and staining the samples used for suberin observation. This procedure was also applied to whole-mount roots with suberized cell layers.

• **Key Results** Clearing of sections results in good image quality to observe the tissue structure and cell walls compared with non-cleared sections. The use of lactic acid as a solvent for fluoro yellow proved superior to previously used solvents such as polyethylene glycol–glycerol. Clearing and fluorescence staining of thin roots such as those of *Arabidopsis thaliana* were successful for suberin visualization in endodermal cells within whole-mount roots. For thicker roots, such as those of maize, sorghum or tea, this procedure could be used for visualizing the exodermis in a longitudinal view. Clearing and staining of peeled maize root segments enabled observation of endodermal cell walls.

• **Conclusions** The clearing procedure using lactic acid improves the quality of images from free-hand sections and clearings. This method enhances the study of plant root anatomy, in particular the histological development and changes of cell walls, when used in combination with fluorescence microscopy.

Key words: Hand sections, Casparian bands, clearing, endodermis, exodermis, fluorescence microscopy.

INTRODUCTION

Free-hand sectioning of living plant tissues often provides an adequate method for rapid and inexpensive microscopic observation of their internal structure. Moreover, this very simple technique often results in high quality images. Other techniques that facilitate sectioning have been published previously (e.g. Ruzin, 1999), including the use of a vibratome or a freezing microtome. An increasing number of papers published over the last few years have images of free-hand-sectioned plant tissues.

Free-hand sectioning has been routinely used in the authors' laboratories, and root structure has been observed, with particular emphasis being paid to the ontogenesis of endo- and exodermal cells (Morita *et al.*, 1996; Lux and Luxová, 2001, 2003/4; Šottníková and Lux, 2003; Lux *et al.*, 2004). The techniques described by Brundrett *et al.* (1988, 1991) influenced many researchers to use fluorescence stains in studies of root apoplastic barriers. By staining lamellar and non-lamellar suberin, these methods allow a clear differentiation between the first and the second

ontogenetic state of endo- and exodermal cells (e.g. Perumalla *et al.*, 1990; Peterson and Perumalla, 1990; Zimmerman and Steudle, 1998; Seago, 2002; Soukup *et al.*, 2002). According to these protocols, the first and second state of endodermal and exodermal development can be distinguished by staining with berberine hemisulphate or fluoro yellow 088 in polyethylene glycol–glycerol, respectively. The amorphous suberin characteristic of Casparian bands can be observed by berberine staining of tissues followed by visualization with UV light microscopy. Lamellar suberin, characteristic of the second stage, can be visualized using UV light microscopy by employing fluoro yellow 088 staining. However, the free-hand sections of various plant organs are often obscure, and the resulting UV or bright-field imaging is of poor quality due to cell contents.

The present study encompassed several goals to improve simple methods for microscopically observing plant structures. One aim was specifically to improve the images offered by free-hand sections of plant organs. Another was to determine whether the original Brundrett *et al.* (1988, 1991) procedures could be changed. The final goal was to establish a whole-mount procedure to observe developmental changes involving exo- and endodermal cells along the root axis.

* This work is dedicated to the 80th anniversary of the birth of the late Dr Mária Luxová.

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MATERIALS AND METHODS

Plant material

Roots of various plant species were used for the study: aerial roots of a greenhouse-grown orchid (*Oncidium* sp.), adventitious roots of hydroponically cultivated melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) (both greenhouse-grown in aerated nutrient solution), nodal roots of field grown sorghum [*Sorghum bicolor* (L.) Moench] and roots of tea plants [*Camellia sinensis* (L.) O.Ktze.] were used. All plants were acquired from the Field Production Science Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo. Additionally, roots of the following plants were used: (a) adventitious roots of onion (*Allium cepa* L.) bulbs obtained from a local store and cultivated in moist vermiculite in a growth chamber at 27 °C with a 14-h day and a light intensity of 20 000 cd sr m⁻²; (b) maize (*Zea mays* L.) primary seminal roots grown under the same conditions as the onion; and (c) *Arabidopsis thaliana* (L.) Heynh ecotype Columbia roots grown from seeds in moist vermiculite containing one-quarter concentration of Hoagland–Arnon nutrient solution (Hoagland and Arnon, 1950) and cultivated in a growth chamber at 20 °C with a 14-h day and a light intensity of 8000 cd sr m⁻². All root samples were washed free of vermiculite or nutrient solution and sectioned fresh, without prior fixation.

Sectioning

Samples for observation were prepared by standard free-hand sectioning (e.g. Ruzin, 1999). The sections were cut with smooth stokes and transferred from the blade into a drop of water on a microscope slide. The water from the drop was then partially absorbed by tissue paper and clearing or staining solution was subsequently added to the sections. Alternatively the Frohlich method of hand sectioning with Parafilm can be used, allowing generation of sections even from thin roots, which cannot be held by hand (Frohlich, 1984).

Preparation of solutions

Clearing solution. Lactic acid (85–92 %, Wako Pure Chemical Industries) was saturated with chloral hydrate (Nacalai Tesque).

Clearing and fluorescence staining solutions. (a) A 0.1 % (w/v) solution of berberine hemisulphate (Sigma) in lactic acid was dissolved at room temperature. (b) A 0.01 % (w/v) solution of fluorol yellow 088 (Sigma) in lactic acid was prepared by heating at 70 °C for 1 h. Due to the low stability of the solution, this was prepared fresh before each use.

Solutions for post-staining. The following stain solutions were prepared: (a) aniline blue (Wako Pure Chemical Industries) 0.5 % (w/v) in distilled water; (b) toluidine blue O (Merck), 0.1 % (w/v) in distilled water; and (c) Safranin O (Kokusan Chemical Works.), 0.5 % (w/v) dissolved in 50 % EtOH.

Staining solutions used in the original procedures of Brundrett et al. (1988, 1991). Fluorescent stains as recommended were prepared as follows: (a) a 0.1 % (w/v) berberine hemisulphate (Sigma) was dissolved in distilled water; and (b) a 0.01 % (w/v) solution of fluorol yellow 088 (Sigma) in polyethylene glycol (PEG; MW 400 Da) was heated at 90 °C for 1 h. An equal volume of 90 % (v/v) glycerol was added to the fluorol yellow–PEG solution.

Clearing and staining procedure

The sections floating in drops of clearing solution on microscope slides were heated over a water bath in covered Petri dishes. Treatment for 1 h, using a high temperature (70 °C), even with large or dense sections, was usually sufficient. The clearing solution was then absorbed by pipette or tissue paper and the sections were thoroughly washed with distilled water added several times to the sections and absorbed by tissue paper. Sections were post-stained and subsequently washed in the same way. Framing by a liquid blocker (PAP pen) proved useful to prevent the loss of sections from slides.

For observation of cell files along the root axis, whole roots were cleared and stained in lactic acid with fluorescence stains (berberine or fluorol yellow) and post-stained with safranin O. This procedure allows observation of epidermal and exodermal cells in thick roots of various species, and in thin roots of *Arabidopsis* it works well for staining and observation of endodermal cells. In thick roots such as those of maize or sorghum, peeling peripheral root tissues exposes the endodermal cells and allows their direct observation. The contrast of cell walls is increased after treatment of peeled samples with berberine in lactic acid. The technique of peeling was originally described by Luxová and Gašparíková (1984) and it has been utilized previously in scanning electron microscopic evaluations of root endodermal silicification in rice, sorghum and bamboo roots (Lux et al., 1999, 2002, 2003a, b).

Photography

Sections were observed with a microscope (Olympus BX51). Ultraviolet illumination was used for fluorescence microscopy (U-MWU2: excitation filter, BP330-385; barrier filter, BA-420; dichroic mirror, DM-400). Images were recorded using a high sensitivity CCD colour camera system (Keyence VB 7010). The software program of this camera system allows generation of an in-focus composite image from up to ten images recorded by merging their sharply focused regions together. Moreover, this program was used to overlay pictures taken of the same sample in up to three different illumination conditions.

RESULTS

Cleared sections

The combination of lactic acid with chloral hydrate proved to be fast and effective for clearing the free-hand sections. Such sections could be mounted directly in the clearing

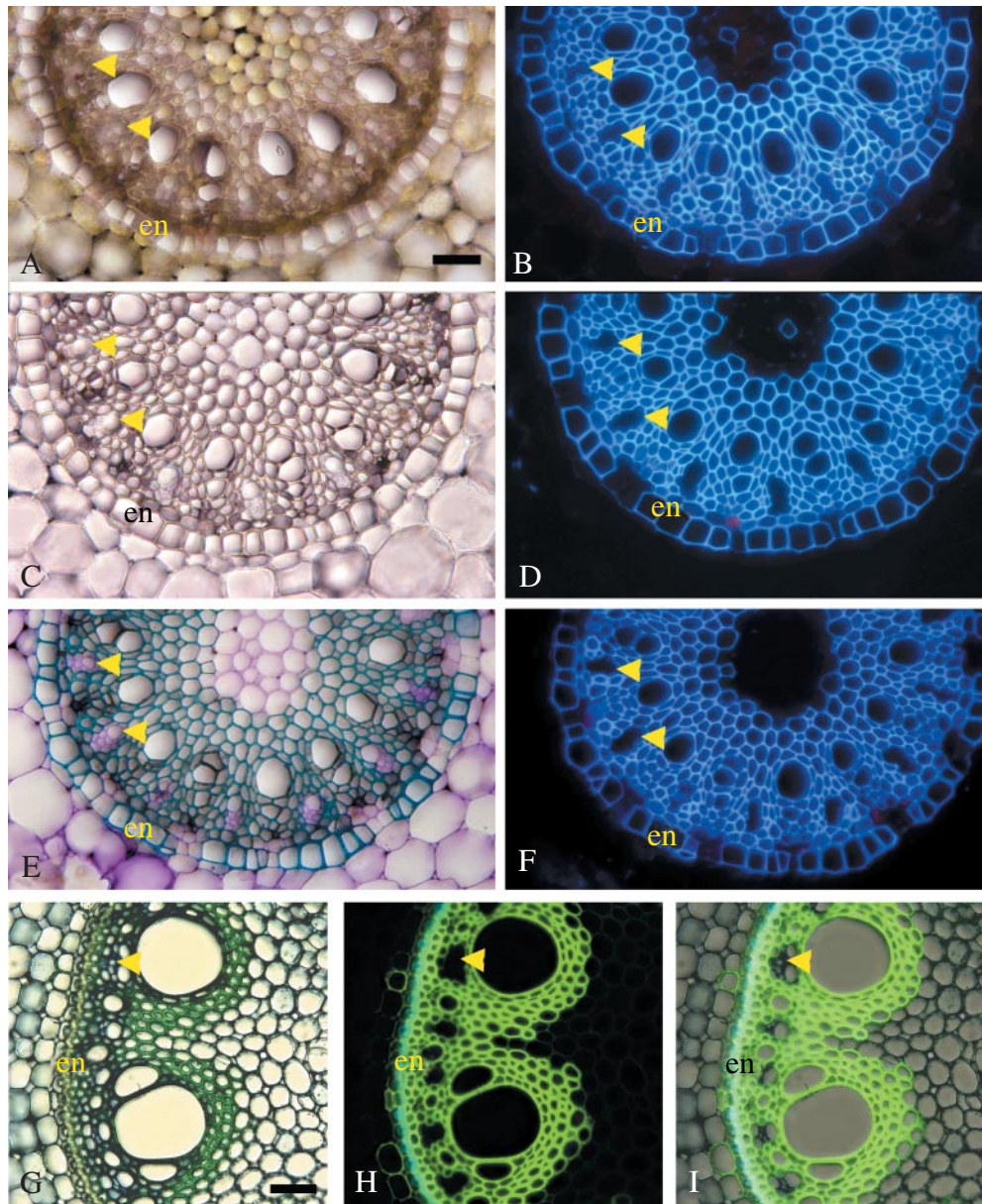


FIG. 1. Free-hand cross-sections of roots with and without clearing viewed with bright field or fluorescence microscopy. (A–F) Samples of aerial root of orchid (*Oncidium* sp.) in white light (A, C and E) and under UV light (B, D and F), without clearing (A and B), after clearing in lactic acid saturated with chloral hydrate (C and D) and with clearing as in (C) and (D), but after staining with toluidine blue (E and F). Note that the phloem poles are clearly visible under bright field after clearing and staining (C and E) and non-distinguishable in fresh sections (A). (G–I) Section of sorghum nodal root after clearing in lactic acid with chloral hydrate and staining with toluidine blue in white light (G), in UV light (H) and the overlapped image of the previous two pictures (I). Scale bars = 50 μ m. Arrowheads indicate phloem poles; en, endodermis.

solution for bright field microscopic observation. For staining or observation in UV light, washing was essential due to the fluorescence of lactic acid. Before covering the sections with a cover glass, it was necessary to check the sections again (by stereomicroscope if necessary) in order to remove all thick or damaged ones. The sections were then viewed after mounting in distilled water or in 50 % glycerol.

Examples of photomicrographs taken of unstained sections of the same sample both with and without clearing are shown in Fig. 1A–F. Cleared sections can be stained to

increase the cell wall contrast in white light. Toluidine blue O or aniline blue was chosen (Fig. 1E), the former usually giving better results. Remarkably good pictures were produced by overlaying images of the same section that were captured under both bright field and fluorescence microscopy (Fig. 1G–I). These combined pictures take advantage of differences in cell wall composition visualized by autofluorescence in UV light, together with images of the structures visible only with bright field microscopy.

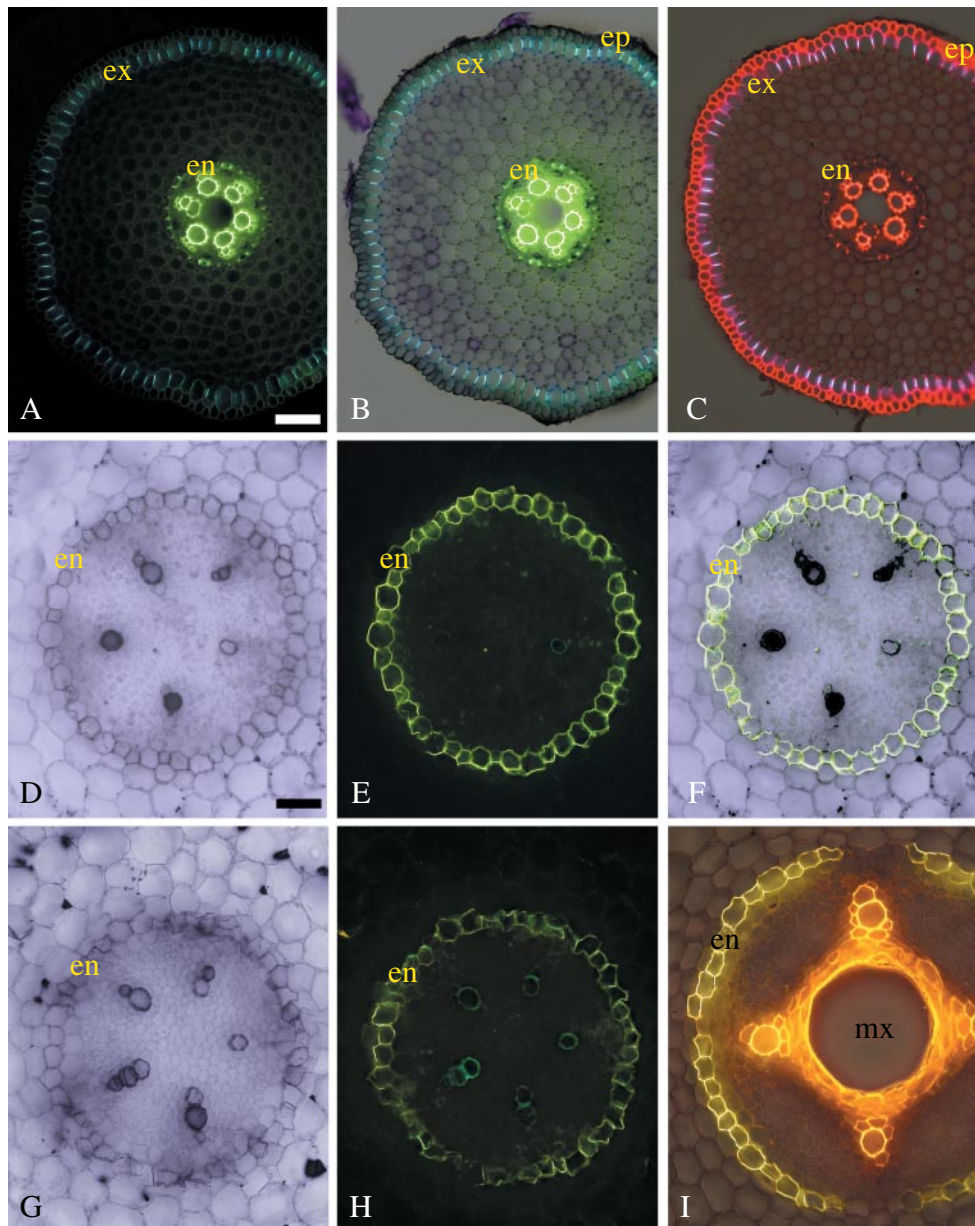


FIG. 2. Free-hand cross-sections of roots with and without clearing, stained with berberine or fluoro yellow: (A–C) roots of onion; (D–H) adventitious roots of melon, (I) root of cucumber. (A, B) The section cleared in lactic acid with berberine, post-stained in an aqueous solution of berberine and with toluidine blue, in UV light (A) and overlapped micrographs in UV light and in white light (B). (C) The section cleared and stained with lactic acid containing berberine and post-stained with safranin, overlapped picture of photos in white light and UV light. (D–F) The same section cleared and stained with lactic acid containing fluoro yellow, in white light (D), in UV light (E) and the overlapped image of the two previous photos (F). Note the contrast of lamellar suberin stained with fluoro yellow and the regular, non-collapsed cells of endodermis. (G, H) The section after staining with fluoro yellow in polyethylene glycol-glycerol. Note irregular shape of partially collapsed endodermal cells. (I) Cucumber adventitious root cleared and stained with lactic acid containing fluoro yellow and post-stained with safranin. ep, epidermis; ex, exodermis; en, endodermis; mx, broad late metaxylem vessel. Scale bars: A = 100 µm (for A–C); D = 50 µm (for D–I).

Observation of amorphous and lamellar suberin in root exo- and endodermis in root cross-sections

Staining of amorphous suberin: Casparian bands. Free-hand sections of roots taken at appropriate distances from the root tip to see the Casparian bands either in exo- or endodermis were prepared and stained for 1 h in berberine hemisulphate solution (0.1 % w/v) dissolved in lactic acid. The sections were washed and post-stained with either

aniline blue as recommended by Brundrett *et al.* (1988) or toluidine blue O. Both stains reduced the autofluorescence of the xylem that sometimes interfered with the contrast of the Casparian bands. Moreover, post-staining results in a clearer view of root tissues under a bright field microscope. In some cases, no post-staining is needed for solely observing Casparian bands. To increase the contrast of Casparian bands in onion root (Fig. 2A and B) after staining in lactic acid with berberine, the sections were washed and

stained again in an aqueous solution of berberine (0.1 % w/v) for an additional 30 min. These same sections were then washed and post-stained with toluidine blue O. Alternatively, safranin O post-staining was used in these root preparations. Similar to previous samples, this post-staining was performed with the addition of a drop of safranin O to the droplet of stain solution (on which sections were floating). Safranin O stains the sections very rapidly, and was therefore usually applied for <1 min. The stain was subsequently washed out and the sections were mounted in water for observation. Safranin O staining defines cell wall structure with a red colour in the white light microscope; under UV light the results of this staining can be variable depending on the composition of the cell wall. In onion, the endodermal Casparian bands were bright red, whereas exodermal Casparian bands appeared light (Fig. 2C).

Staining of lamellar suberin. Root sections of various plants prepared as described above were stained with drops of fluorol yellow 088 (0.01 % w/v) in lactic acid. The same procedure used for berberine staining was performed and the sections were viewed using a fluorescence microscope. A comparison of suberin lamella staining produced using the modified lactic acid–fluorol yellow solution to the standard fluorol yellow–polyethylene glycol–glycerol procedure is shown in Fig. 2D–H. Merging of pictures taken under both bright field and fluorescence microscopy is also very useful in this case for fully visualizing the root structure (Fig. 2F). Post-staining with safranin O combined with the lactic acid–fluorol yellow procedure also resulted in better-defined images (Fig. 2I).

Observation of cell files along the root axis

Epidermal and exodermal cells of maize and tea. Whole roots of maize were cleared with lactic acid saturated with chloral hydrate. Treatment of seminal maize roots in this solution in Petri dishes at 70 °C for 1 h was sufficient; however, the treatment time might differ for various types of roots and different species. The roots were then washed and stained with berberine dissolved in lactic acid for 30 min at room temperature, and briefly post-stained with safranin O and aniline blue. These stains increased the contrast of epidermal cell walls for bright field microscopy and at the same time reduced their autofluorescence when viewed under UV illumination. Roots were arranged in a zig-zag pattern on the microscope slides and were covered by a large cover glass, allowing for observation and measurement of epidermal cells in a bright field (Fig. 3A). After switching to UV illumination, a clear image of the fluorescent exodermis allowed observation and measurement of its component cells (Fig. 3B).

In tea roots, a different procedure yielded superior results. In this case, whole roots were cleared and simultaneously stained with fluorol yellow dissolved in lactic acid at 70 °C for 1 h and briefly post-stained with safranin O. This procedure resulted in high contrast and very clear images of the exodermal network using UV light illumination, as well as highlighting the epidermal cells under white light (Fig. 3C).

Endodermal cells of intact arabidopsis roots. Roots of arabidopsis were stained with 0.01 % fluorol yellow in lactic acid in Petri dishes at 70 °C over a water bath for 1 h. The roots were then washed and either post-stained in safranin O and washed again, or directly observed without post-staining. The thin roots of this species contain only two layers of cells external to the endodermis (epidermis plus the outer cortical layer). This allows direct observation of the endodermis using fluorescence microscopy, as the endodermal cells impregnated by lamellar suberin are clearly visible under UV light (Fig. 3D–F). The contrast of the suberin staining is about the same with or without safranin O post-staining, with the exception of the colour enhancement provided by the post-stain. In roots treated with fluorol yellow in lactic acid, the colour of lamellar suberin is yellow whereas after safranin O post-staining this material turns red (not shown).

Endodermal cells of peeled maize and sorghum roots. The relatively thick maize and sorghum roots with their multi-layered cortices do not allow for direct observation of endodermal cells in cleared, whole roots. Thus, these roots were manually peeled—the peripheral root tissues were carefully picked using a pair of forceps, and then pulled to separate the endodermis covering the stele. The peeled root samples were stained for 1 h in berberine (0.1 % w/v) dissolved in lactic acid in Petri dishes at 70 °C. The staining increased the contrast of radial and transverse endodermal cell walls in UV light (Fig. 3G).

DISCUSSION

Clearing of sections

Clearing techniques have been used for decades in plant microtechnique for preparing whole-mount specimens. Recently, some clearing methods were developed for use in whole-mount *in situ* hybridization (Bauwens *et al.*, 1994; Engler *et al.*, 1994) and for specimens with immunolabelled microtubules and microfilaments (in the whole-mount rye root tips; Erickson and Carter, 1996). However, such clearing techniques were infrequently utilized for hand-sectioned plant material. Among the exceptions are the studies conducted by Peterson and co-workers (e.g. Peterson *et al.*, 1982; Weerdenburg and Peterson, 1984; Perumalla and Peterson, 1986; Peterson and Lefcourt, 1990). The authors used free-hand cross-sections cleared overnight in 1 % (w/v) NaOH and stained with *Chelidonium majus* root extract for their studies of endo- and exodermis by fluorescence microscopy. Clearing of hand sections is also used to examine mycorrhizal roots (e.g. Brundrett *et al.*, 1990).

Clearing of the sections on the microscope slide works well not only with root samples but also with various other plant organs such as stems, leaves, etc. (e.g. *Clematis* stem Fig. 3H and I). Clearing also works well in mesh baskets as proposed in Brundrett and Kendrick (1990) and Brundrett *et al.* (1990). The staining of cleared sections is possible with several stains. Toluidine blue O, aniline blue and safranin O have been used successfully, although several other stains may perform equally well. The clearing solution

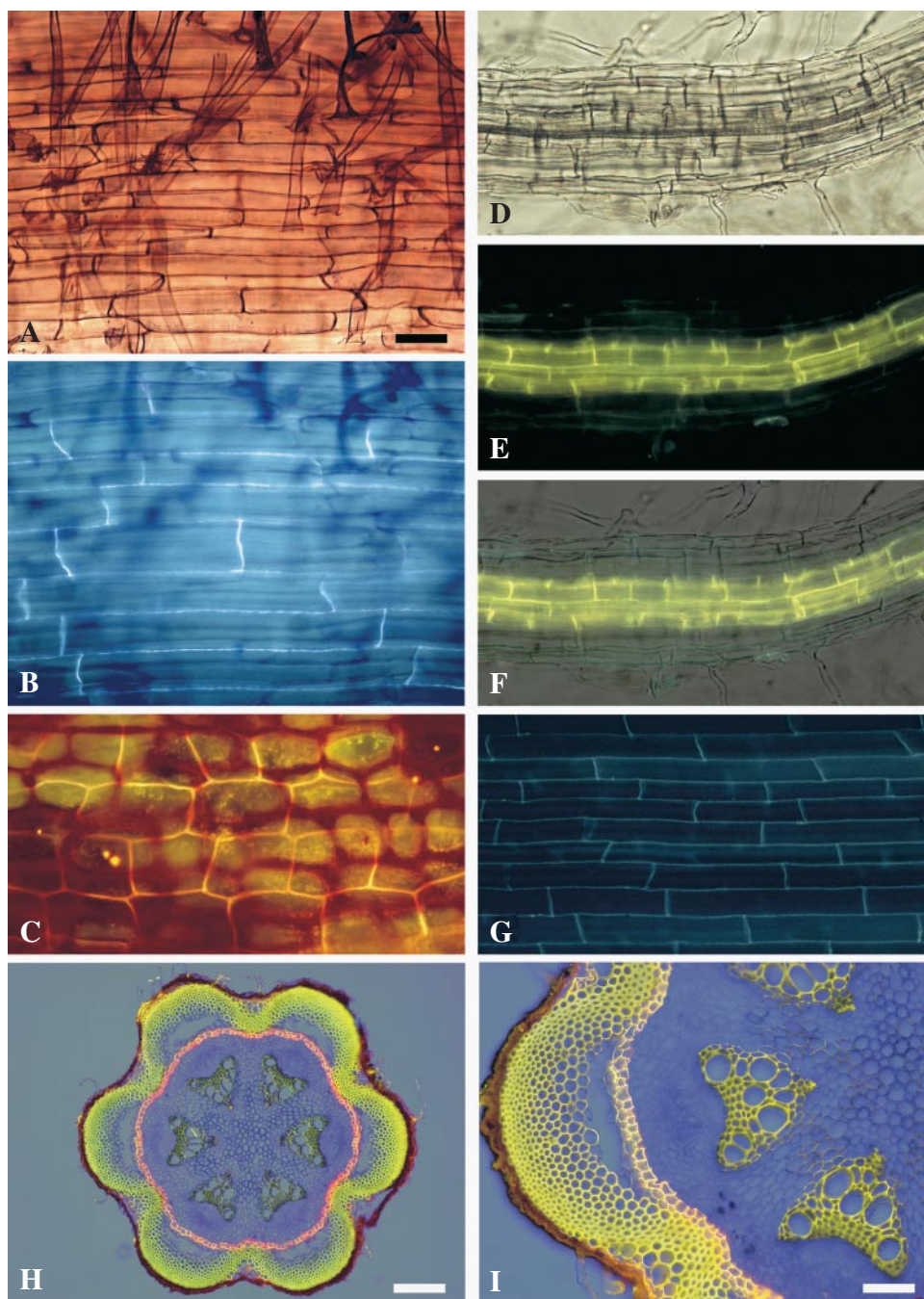


FIG. 3. (A–G) Longitudinal views of roots in fluorescence microscopy; (H–I) free-hand stem cross-section with clearing. (A, B) Surface view of maize seminal root after clearing in lactic acid with chloral hydrate, staining with aqueous solution of berberine and post-stained with safranin; focused on epidermal cells in white light; (B) the same area as in (A) but focused on exodermal cells in UV light. (C) Tea root after clearing and staining in lactic acid containing fluoro yellow, and post-staining with safranin in UV light. (D–F) Root of arabidopsis after clearing and staining in lactic acid containing fluoro yellow in white light (D), in UV light (E) and overlapped picture after combining both previous images (F). Note the clear image of bright yellow-stained endodermis visible through the outer cortical and epidermal layers. (G) Endodermal network of sorghum nodal root in surface view in UV light. The sample was peeled (to remove the outer cortical layers and visualize the surface of the stele covered by endodermal cells), cleared and stained in lactic acid containing berberine. (H, I) Cross-section of *Clematis* stem after clearing in lactic acid saturated with chloral hydrate as overlapped images taken with white light and UV light.

Scale bars: A = 50 μ m (for A–G); H = 200 μ m; I = 100 μ m.

itself, as well as the stains used, significantly diminish the intensity of cell wall autofluorescence, a complication which might prove problematic in some cases. This is true especially for toluidine blue O as it causes much greater

attenuation of the autofluorescence than aniline blue. In any case, cleared sections, either stained or unstained, are usually superior to non-cleared ones for imaging with bright-field light microscopy.

Staining of suberin in root cross sections

This procedure is a modification of the staining procedure originally described by Brundrett *et al.* (1988, 1991). These excellent protocols for suberin staining have enabled the detailed study of exo- and endodermal ontogenesis. Their popularity is evident in the citation indexes of these papers (119 and 71 times cited, respectively; data are as of March 2005 according to Web of Science[®], Thomas Corporation). It was found that both fluorescent stains—berberine and fluorol yellow—can be applied to sections when dissolved in lactic acid, so that simultaneous staining and clearing is possible. Alternatives for post-staining after berberine treatment include aniline blue, toluidine blue O or safranin O. A post-staining treatment results in excellent contrast enhancement of Casparian bands against the rest of the cell wall for some specimens (e.g. exodermal Casparian bands in onion). Cell damage in delicate melon roots from hydroponics culture caused by the original fluorol yellow solution of Brundrett *et al.* (1991) can be reduced by the use of the fluorol yellow dissolved in lactic acid (Fig. 2D–H). Such damage may have resulted from tissue shrinking caused by high osmotic pressure of the staining solution. The faster permeability of lactic acid due to its lower molecular size than PEG might reduce the osmotic gradient between the inside and outside of the cells, hence reducing the occurrence of structural damage to the cells during the clearing/staining process.

Observation of cell files in whole-mount roots

When root cells in longitudinal view are studied, sectioning of embedded root segments is very laborious and time-consuming. Also, longitudinal sections only provide information for short distances along the roots. To allow for observation of cell length among the root tissues, two techniques were employed. These techniques facilitate observation and measurement of three root cell layers—epidermis, exodermis and endodermis—without sectioning; the development of the exo- and endodermis can also be studied. The procedure was successful for maize and sorghum roots and might also be useful for other cereal plants. In another species (tea roots), observation was possible for only epidermal and exodermal cells. The first technique involves clearing intact roots followed by microscopic observation of epidermal and exodermal cells using white or UV light alternately. The other technique requiring peeling of roots is based on the fact that softer peripheral cortical cells can be separated from the thick-walled, mature endodermis covering the stele in some species. This process was successful in grasses with endodermal cells exhibiting intensively thickened secondary walls and can be applied only to older roots or root parts, since segments close to the apex cannot be peeled.

Thin roots of arabidopsis are the subject of studies detailed in numerous recent papers (e.g. Dolan *et al.*, 1994; Baum *et al.*, 2002). However, development of the endodermis in arabidopsis is still not completely elucidated. One of the reasons is that the use of cross-sections for staining and visualization of changes in endodermal cell

wall composition is very difficult in these thin roots. Clearing combined with fluorol yellow staining facilitates the direct observation of endodermal cell files along the root axis of the whole mount arabidopsis root. Similar to the procedure described here, methods to produce cleared whole mount specimens of arabidopsis hypocotyls were recently published in a technical note by Homma and Karahara (2004). The authors employed treatment in 10% (w/v) KOH at 105 °C for 1 min to visualize Casparian bands and xylem vessels by autofluorescence to construct a three-dimensional model illustrating hypocotyl structure.

The staining properties of Casparian bands and suberin lamellae in exo- and endodermal cells depend on the composition of their cell walls. This feature is highly variable among different species and can vary even within the same species during ontogenesis. Therefore, some modification of staining procedures, including changes in incubation times or modified post-staining procedures, might be necessary to optimize the conditions for a given specimen.

ACKNOWLEDGEMENTS

A.L. acknowledges the hospitality extended by the staff at the Field Production Science Center at the University of Tokyo during his stay as a Visiting Professor. This work was partially supported by grant 1/0100/03 from the Slovak Grant Agency VEGA and COST Action 859. The authors appreciate the generosity of the Keyence Company in allowing us to use their CCD colour camera to take photos for this paper. For seeds and plant materials we are thankful to Dr Y. Mine, Dr J. Kyojuka (The University of Tokyo) and Professor E. Tanimoto (Nagoya City University). The authors appreciate the valuable comments on and improvements to the manuscript by the referees and the decision editor.

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